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Caseinolytic and milk-clotting activities from *Moringa oleifera* flowers

Emmanuel V. Pontual [1], Belany E.A. Carvalho [1], Ranilson S. Bezerra, Luana C.B.B. Coelho, Thiago H. Napoleão, Patrícia M.G. Paiva *

Departamento de Bioquímica-CCB, Universidade Federal de Pernambuco, Cidade Universitária, 50670-420 Recife, Pernambuco, Brazil

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ABSTRACT

This work reports the detection and characterization of caseinolytic and milk-clotting activities from *Moringa oleifera* flowers. Proteins extracted from flowers were precipitated with 60% ammonium sulphate. Caseinolytic activity of the precipitated protein fraction (PP) was assessed using azocasein, as well as α_s-, β- and κ-caseins as substrates. Milk-clotting activity was analysed using skim milk. The effects of heating (30–100 °C) and pH (3.0–11.0) on enzyme activities were determined. Highest caseinolytic activity on azocasein was detected after previous incubation of PP at pH 4.0 and after heating at 50 °C. Milk-clotting activity, detected only in the presence of $CaCl_2$, was highest at incubation of PP at pH 3.0 and remained stable up to 50 °C. The pre-treatment of milk at 70 °C resulted in highest clotting activity. Enzyme assays in presence of protease inhibitors indicated the presence of aspartic, cysteine, serine and metallo proteases. Aspartic proteases appear to be the main enzymes involved in milk-clotting activity. PP promoted extensive cleavage of κ-casein and low level of α_s- and β-caseins hydrolysis. The milk-clotting activity indicates the application of *M. oleifera* flowers in dairy industry.

1. Introduction

Plant proteases, enzymes that catalyse the hydrolysis of peptide bonds, participate in several biological processes, including mobilisation of storage proteins, degradation of light-damaged chloroplast proteins, defense against phytopathogen attack, tissue differentiation, and floral senescence (Estelle, 2001). Different industrial processes utilise proteases such as papain, bromelain, and ficin, and new enzymes with appealing physicochemical properties have been investigated for that purpose (Feijoo-Siota & Villa, 2001).

Clotting of milk is a result of the action of proteases that destabilize casein micelles, which are particles present in fresh milk dispersed in a continuous phase comprising water, salt, lactose and whey proteins (Kruif, 1999). The caseins α_s and β are localised within the micelle, whose structure is maintained in solution by the κ-casein hydrophilic domain (Lo Piero, Puglisi, & Petrone, 2002). The hydrolysis of κ-casein results in the collapse of micelles and exposure of α_s- and β-caseins to calcium, leading to separation of milk into a solid (clot or curd) and liquid (whey) phases (Abreu, 2005).

In cheese production, milk-clotting by calf rennet is the procedure most commonly used. However, the low supply of calf rennet and the incidence of bovine spongiform encephalopathy are incentives in the search for enzymes from microorganisms and plants (Ahmed, Morishima, Babiker, & Mori, 2009; Barbano & Rasmussen, 1992; Cavalcanti, Teixeira, Lima Filho, & Porto, 2004; Shieh, Thi, & Shih, 2009).

An early study showed that the cheese produced using extract from *Calotropis procera* leaves was harder, less cohesive and gummier than that obtained using acidic pH as clotting agent (Aworh & Muller, 1987). Bruno, Lazza, Errasti, López, Caffini, and Pardo (2010) reported that the cheese produced using extract from *Bromelia hieronymi* fruits was acceptable in appearance, body, texture, and flavour. The *Albizia julibrissin* seed extract was also used as milk-clotting agent, and the resulting cheese did not develop bitterness after three months of ripening (Otani, Matsumori, & Hosono, 1991). Extract from *Cynara cardunculus* flowers containing proteases (cyprosins) is traditionally used in artisanal production of cheeses, and the recombinant form of cyprosin B is available for large-scale use (Sampaio, Fortes, Cabral, Pais, & Fonseca, 2008).

Milk-clotting activities from plant preparations have been associated with serine and aspartic proteases. A serine protease of *Cucumis melo* fruit exhibited a more stable milk-clotting activity, when compared to that of papain (Uchikoba & Kaneda, 1996). Additionally, it has been reported that a serine protease from *Lactuca sativa* leaves promoted clotting of skim milk as well as of milk with different fat contents (Lo Piero et al., 2002; Uchikoba & Kaneda, 1996). Aspartic protease from *Oryza sativa* seeds promoted cleavage of κ-casein, in a pattern similar to that obtained with chymosin and pepsin (Asakura, Watanabe, Abe, & Arai, 1997), and aspartic proteases from extract of *Silybum marianum* flowers

* Corresponding author. Tel.: +55 8121268540; fax: +55 8121268576.
E-mail address: ppaivaufpe@yahoo.com.br (P.M.G. Paiva).
[1] These authors equally contributed to this study

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hydrolysed caprine and ovine milk caseins (Cavalli, Silva, Cimino, Malcata, & Priolo, 2008).

Flowers of *Moringa oleifera* (Moringaceae family) are rich in calcium, potassium and antioxidants (α and γ-tocopherol), and are used in human diet, mainly in the Philippines (Makkar & Becker, 1996; Ramachandran, Peter, & Gopalakrishnan, 1980; Sánchez-Machado, López-Cervantes, & Vázquez, 2006). This work reports the detection in *M. oleifera* flowers of caseinolytic and milk-clotting activities using azocasein and skim milk as substrates, respectively. The effects of pH, temperature and protease inhibitors on these enzyme activities are also reported. Additionally, the caseinolytic and milk-clotting activities were assayed using α_s-, β- and κ-caseins or heated skim milk as substrates, respectively.

2. Materials and methods

2.1. Plant material

M. oleifera Lam. (Eudicots, Eurosids II, Order Brassicales, Family Moringaceae) has the vernacular names "moringa" in Portuguese, "árbol del ben" in Spanish and horseradish tree in English. Flowers were collected in Recife City, State of Pernambuco, northeastern Brazil. A voucher specimen is archived under number 73,345 at the herbarium Dárdano de Andrade Lima (Instituto Agronômico de Pernambuco, Recife, Brazil). The flowers were detached from the inflorescence rachis at the pedicel and dried at 27 ± 2 °C, relative humidity of 70 ± 5%, for 7 days before use. The extraction procedure is described below.

2.2. M. oleifera flower preparations

Powder (20 mesh) of *M. oleifera* dried flowers (50 g) was suspended in 0.15 M NaCl (500 ml) and homogenised in magnetic stirrer (4 h at 4 °C). After filtration through gauze and centrifugation (9,000 g, 15 min, 4 °C), the flower extract (clear supernatant) was treated with ammonium sulphate at 60% saturation (Green & Hughes, 1955). The precipitated protein fraction (PP) collected by centrifugation and the 60% supernatant fraction were dialysed (10 ml; 3.5 kDa cut-off membrane) against distilled water (4 h) and 0.15 M NaCl (2 h) using a volume of 2 L for dialysis fluid.

2.3. Protein content

Protein concentration was determined according to Lowry, Rosebrough, Farr, and Randall (1951) using serum albumin (31–500 μg/ml) as standard.

2.4. Caseinolytic activity

Caseinolytic activity was determined using azocasein (Sigma–Aldrich, USA) as substrate, according to Azeez, Sane, Bhatnagar, and Nath (2007). Flower extract (100 μl, 3.0 mg of protein), PP (100 μl, 3.2 mg of protein) or 60% supernatant fraction (100 μl, 3.0 mg of protein) was mixed with 300 μl of 0.1 M sodium phosphate pH 7.5 containing 0.6% (w/v) azocasein. The mixture was supplemented with 100 μl of 0.1% (v/v) Triton X-100 and incubated at 37 °C for 3 h. The reaction was stopped by adding 200 μl of 10% (w/v) trichloroacetic acid, and after incubation (4 °C, 30 min) the mixture was centrifuged at 9,000 g for 10 min. Next, the absorbance at 366 nm of the supernatant was determined.

Caseinolytic activity was also determined according to Sousa and Malcata (1998) using bovine α_s-, β-, and κ-caseins purchased from Sigma–Aldrich, USA. PP (50 μl, 1.7 mg of protein) was added to α_s-, β- or κ-casein solutions (1 ml, 10 mg of protein) in 0.1 M sodium phosphate buffer, pH 6.5 and reaction was allowed to proceed at 37 °C. Aliquots of 10 and 900 μl from the reaction mixtures were retrieved within 10, 30, 60, 120 min and 24 h of incubation. The aliquots of 10 μl were heated at 100 °C for 5 min and submitted to SDS–PAGE as described in Section 2.5. The aliquots of 900 μl were evaluated for absorbance at 366 nm after addition of 10% (w/v) trichloroacetic acid (200 μl) and centrifugation (9,000 *g*, 10 min, 4 °C). One unit of caseinolytic activity was defined as the amount of enzyme that promoted a 0.01 increase in absorbance. Chymosin (50 μl, 10 μg; Chy-Max® Liquid, Chr. Hansen, Denmark) and 0.15 M NaCl were used as positive and negative controls, respectively.

2.5. Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (SDS–PAGE)

Hydrolysis of α_s-, β- or κ-caseins by PP and chymosin (positive control) were evaluated by SDS–PAGE using 15% (w/v) polyacrylamide gels (Laemmli, 1970). Aliquots (10 μl) from reaction mixtures described in the Section 2.4, and molecular mass markers (SigmaMarker™ kit from Sigma–Aldrich, USA, containing the standard proteins: bovine serum albumin, 66,000 Da; glutamic dehydrogenase from bovine liver, 55,000 Da; ovalbumin from chicken egg, 45,000 Da,; glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle, 36,000 Da; carbonic anhydrase from bovine erythrocytes, 29,000 Da; trypsinogen from bovine pancreas, 24,000 Da; trypsin inhibitor from soybean, 20,000 Da; α-lactalbumin from bovine milk, 14,200 Da; and aprotinin from bovine lung, 6,500 Da) were applied on gel. After running and staining with 0.02% (v/v) Coomassie Brilliant Blue in 10% acetic acid, the gels were dehydrated and scanned. The densitograms were obtained using the software Scion Image Beta 4.02.2 (Scion Corporation, Frederick, MD, USA) and indicated the intensity of polypeptide bands.

2.6. Milk-clotting activity

The substrate (10% skim milk, Molico®, Nestlé, Brazil) was prepared in distilled water or in 10 mM $CaCl_2$ in water, and pH was adjusted at 6.5. The milk (2.0 ml) was incubated with flower extract (0.3 ml, 9.0 mg of protein), PP (0.3 ml, 9.8 mg of protein) or 60% supernatant fraction (0.3 ml, 9.0 mg of protein) at 37 °C, and curd formation was observed. The end point was recorded when the full separation between whey and curd was observed. One milk-clotting unit was defined as the amount of enzyme that clots 2 ml of the substrate within 180 min. Chymosin and 0.15 M NaCl were used as positive and negative controls, respectively. Milk-clotting activity was also determined using skim milk (10% w/v) heated at 30, 50 and 70 °C.

2.7. Effect of heating, pH and protease inhibitors on caseinolytic and milk-clotting activities

Caseinolytic (on azocasein) and milk-clotting activities were determined after heating (30 min) of PP at 30, 40, 50, 60, 70, 80, 90 and 100 °C under the same conditions described in Sections 2.4 and 2.6, respectively.

To determine the effect of pH on the enzyme activities, PP was previously incubated in 0.1 M citrate phosphate buffer (pH 3.0 to 6.0, 24 h, 37 °C), 0.1 M sodium phosphate pH 7.0, 0.1 M Tris–HCl (pH 8.0 and 9.0) or 0.1 M sodium borate buffer (pH 10.0 and 11.0). Next, assays were performed as described in Sections 2.4 and 2.6.

Inhibitors (8 mM, 1 ml) of serine proteases (phenylmethylsulfonyl fluoride, PMSF), cysteine proteases (transepoxy-succinyl-leucyl-amido-(4-guanidino)-butane; E-64), metallo proteases (ethylenediaminetetracetic acid, EDTA), and aspartic proteases (pepstatin A) were added to PP (1 ml, 32 mg of protein) and the

mixture was incubated at 37 °C for 30 min. Subsequently, the incubation mixtures were evaluated for caseinolytic (on azocasein) and milk-clotting activities. Inhibition percentages were calculated as follows: % inhibition = 100 − [100 × (residual activity/activity in control without inhibitor)].

2.8. Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA), and data were expressed as a mean of replicates ±SD. Significant differences between treatment groups were analysed by the Studenťs t-test (significance at $p < 0.05$) using the Origin 6.0 program.

3. Results and discussion

3.1. Caseinolytic and milk-clotting activities from M. oleifera flowers

Flower extract (2,940 mg of protein) was not able to hydrolyse azocasein, and it did not show milk-clotting activity using milk supplemented or not with $CaCl_2$. Differently, Satish, Sairam, Ahmed, and Urooj (2012) reported that aqueous extracts from *M. oleifera* leaf and roots showed caseinolytic activity and were also able to hydrolyse human plasma clot. Although proteolytic activity was not detected in flower extract, PP (480 mg of protein) showed caseinolytic (37.5 U, using azocasein) and milk-clotting (1.9 U, using milk supplemented with $CaCl_2$) activities. Fig. 1 shows the aspect of milk-clotting activity in the assay tubes. The 60% supernatant fraction (2,460 mg of protein) hydrolysed azocasein (1.4 U), but it did not show milk-clotting activity. The data reveal that ammonium sulphate concentrated the caseinolytic and milk-clotting activities from *M. oleifera* flowers in PP. Milk-clotting enzymes of extracts of *Albizia lebbeck*, *Helianthus annus* and *Solanum dubium* seeds were also precipitated using ammonium sulphate (Ahmed, Babiker, & Mori, 2010; Egito et al., 2007). According to Kent (1999) protein concentration using ammonium sulphate has three main advantages: it is a rapid and inexpensive method, it does not affect the structure and function of proteins, and the salt can be easily removed from the protein solution by dialysis.

Fig. 1. Milk-clotting activity of positive control chymosin (1), negative control 0.15 M NaCl (2) and PP (3).

Milk-clotting activity from PP was $CaCl_2$-dependent, similarly to what has been reported for *Solanum dubium* and *Withania coagulans* seeds, *Bromelia hieronymi* fruits and *Cynara scolymus* flowers (Ahmed et al., 2010; Bruno et al., 2010; Chazarra, Sidrach, López-Molina, & Rodríguez-López, 2007; Naz, Masud, & Nawaz, 2009). $CaCl_2$ forms bridges between positive and negative charges on casein micelles, causing them to break and releasing α_s- and β-caseins; curd is formed due to association between these proteins and calcium (Abreu, 2005; Ahmed et al., 2010; Anema, Lee, & Klostermeyer, 2005; Ishak et al., 2006).

Milk-clotting activity exerted by PP did not change when milk was heated up to 30 and 50 °C. However, the activity using milk heated up to 70 °C as substrate was higher (3.6 U) than when non-heated milk (1.8 U) was used. Similarly, the milk-clotting activities from goat (*Capra hircus*) chymosin and *C. scolymus* flower extracts have been reported to reach the highest value when the milk was heated up to temperatures above 50 °C (Chazarra et al., 2007; Kumar, Sharma, Mohanty, Grover, & Batish, 2006). Protein aggregation by heating of milk has been related to the increasing of milk clotting activity (Nájera, Renobales, & Barron, 2003).

Bovine α_s-, β-, and κ-caseins were used as substrates to determine the specificity of caseinolytic activity from PP. The enzyme reactions were monitored by absorbance at 366 nm. Fig. 2A shows that hydrolysis of κ-casein by PP started after 30 min of incubation, while degradation of α_s- and β-casein could only be detected after 60 min. Incubation for longer periods (120 min and 24 h) did not lead to any considerable improvement in degradation of α_s- and β-caseins by PP, though hydrolysis of κ-casein increased over 4 times (Fig. 2A). Oppositely, milk-clotting enzymes from *C. cardunculus* flowers have been reported to hydrolyse α_s-casein better than β-casein, and was less effective in cleaving κ-casein (Ordiales et al., 2012).

Chymosin is the major enzyme of calf rennet, and it has been extensively used in the dairy industry to produce a stable curd with good flavour due to its high specificity for the κ-casein (Rao, Tanksale, Ghatge, & Deshpande, 1998). Thus, this enzyme was used as a benchmark positive control. Specificity of PP for bovine caseins was similar to that of chymosin, which extensively cleaved κ-casein and promoted very slight hydrolysis of α_s- and β-caseins (Fig. 2B). On the other hand, the time course of κ-casein hydrolysis by PP was slower than that by chymosin (Fig. 2). However, unlike chymosin, PP is a partially purified protease preparation and thus the protein concentration reflects the amount of flower extract proteins that were precipitated with ammonium sulphate.

The molecular masses of bovine α_s-, β-, and κ-caseins on SDS–PAGE were between 20 and 25 kDa (Fig. 3), values that were similar to those reported by Dalgleish (1990). The degrees of casein hydrolysis by PP and chymosin were also evaluated by the reduction of α_s-, β-, and κ-caseins bands on SDS–PAGE, since peptides from casein proteolysis can be quantified by gel scanning, followed by densitometry (Cavalli et al., 2008; Franco, Prieto, Urdiales, Fresno, & Carballo, 2001).

The densitogram revealed that the intensities of α_s-casein bands (Fig. 3A, lanes 1 and 2) did not fall after incubation with PP for 10 to 120 min. The intensities of α_s-casein bands were reduced only after 24 h (Fig. 3A, lanes 1 and 2), indicating the occurrence of hydrolysis with the generation of a remarkably intense 17-kDa polypeptide band (Fig. 3A, lane 3). The peak in the densitogram for α_s-casein band after incubation with positive control chymosin was higher than that obtained after incubation with PP (Fig. 3A, lanes 1 and 2), indicating that α_s-casein was more hydrolysed by PP than by chymosin. Low reduction of β-casein band intensity was observed only after 24-h incubation with PP and chymosin (Fig. 3B, lane 1). Hydrolysis by PP generated several polypeptides with molecular mass between 7 and 19 kDa (Fig. 3B,

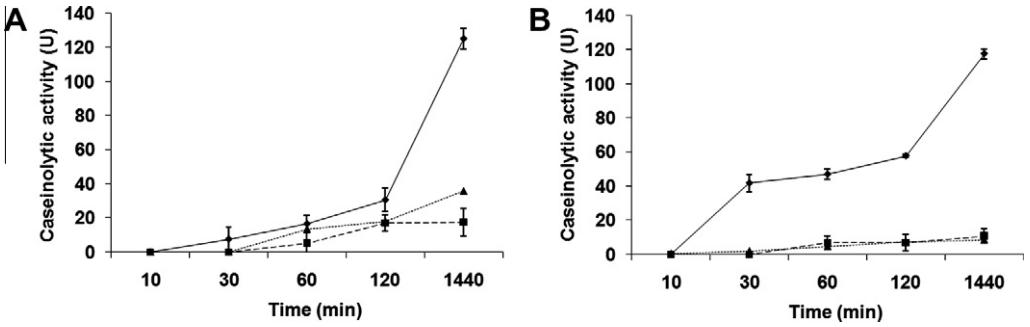

Fig. 2. Time course of hydrolysis of bovine α_s- (■), β- (▲) and κ- (♦) caseins by PP (A) and positive control chymosin (B).

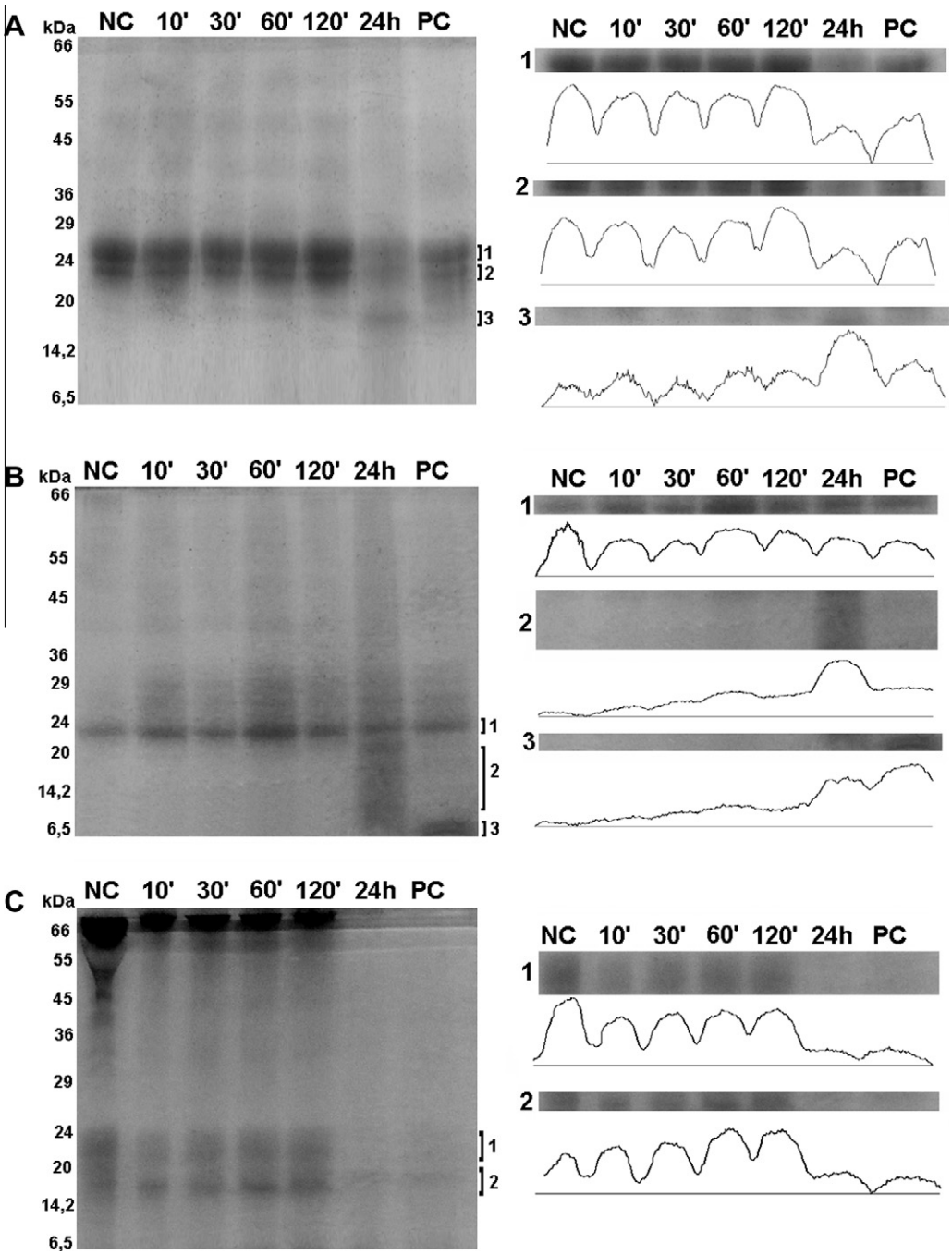

Fig. 3. SDS–PAGE patterns of hydrolysis of α_s- (A), β- (B) and κ- (C) caseins by PP for 10, 30, 60, 120 min and 24 h or by chymosin (PC, positive control) for 24 h. 0.15 M NaCl was used as negative control (NC). The gels were stained with Coomassie Brilliant Blue and the intensities of the polypeptide bands are represented in the densitograms obtained after gel scanning and analysis using Scion Image Beta 4.02.2 software.

lane 2), while cleavage by chymosin resulted mainly in polypeptides with very low molecular masses (Fig. 3B, lane 3). Reduction in intensity of κ-casein band due to hydrolysis by PP after 10, 30, 60 and 120 min (Fig. 3C, lane 1) was accompanied by an increase in the intensity of a 16-kDa polypeptide band, which probably corresponds to para-κ-casein (Fig. 3C, lane 2). No other peak of intensity in the region of κ-casein band was detected after incubation with PP and chymosin for 24 h, revealing total degradation of protein (Fig. 3C, lane 1). In addition, the para-κ-casein band intensity was strongly reduced after 24-h incubation with PP and chymosin (Fig. 3C, lane 2). Chymosin cleaves a single peptide bond in κ-casein, producing insoluble para-κ-casein and a C-terminal glycopeptides (Fox & Stepaniak, 1993; Rao et al., 1998). Extracts from sunflower (*Helianthus annuus*), as well as from albizia (*Albizia lebbeck*) and *S. dubium* seeds, have been proved to hydrolyse κ-casein to para-κ-casein (Ahmed et al., 2010; Egito et al., 2007).

Curd constituents include α_s-, β- and para-κ-caseins (Abreu, 2005). The detection of para-κ-casein on SDS–PAGE after casein hydrolysis by PP and the fact that milk-clotting activity of PP was detected only in the presence of calcium suggests that milk coagulation was probably due to the degradation of κ-casein, leading to the collapse of the micellar structure and aggregation of α_s- and β-caseins under the influence of calcium, resulting in gel formation (Merin, Talpaz, & Fishman, 1989).

PP from *M. oleifera* flowers is a potentially useful tool in cheese production processes, since it did not promote extensive hydrolysis of α_s- and β-caseins. The speed of hydrolysis of caseins influences the yield, consistency as well as flavour of cheese, and slow degradation of α_s- and β-caseins is guarantee of production of a firm curd, which is what occurs when chymosin is used, as mentioned above (Bruno et al., 2010; Fox, 1989). Plant rennets which promote extensive proteolysis of caseins are inappropriate for cheese production, because the generated peptides confer a bitter taste (Lo Piero et al., 2002; Macedo, Faro, & Pires, 1996).

3.2. Effect of heating, pH and protease inhibitors on caseinolytic and milk-clotting activities

Caseinolytic activity on azocasein significantly increased after heating of PP at 50 °C, while loss of this activity was detected after heating of PP at 60 °C (Table 1). Caseinolytic activities from *Lactuca sativa* leaves, *Opuntia ficus-indica* fruits and *S. dubium* seeds were also shown to be highest at 50, 55 and 70 °C, respectively (Ahmed et al., 2009; Lo Piero et al., 2002; Teixeira, Santana, Pais, & Clemente, 2000). Molecular rearrangements in protein structure can lead to increase of enzyme activity (Purich, 2010).

Caseinolytic activity was higher when PP was previously incubated at pH 4.0 and 7.0 (Table 2). A partially purified enzyme from *S. dubium* seeds also showed proteolytic activity towards azocasein at pH 4.0 but, unlike *M. oleifera* activity, the enzyme was highly active up to pH 11.0 (Ahmed et al., 2009). It is known that pH affects the shape, charge properties, the correct positioning of the substrate and the ionisation of side chains of amino acids, in both the active site and in the whole enzyme (Purich, 2010).

Heating of PP from 30 to 40 °C did not interfere in milk-clotting activity, which increased significantly after heating at 50 °C and was neutralised at 70 °C (Table 1). Milk-clotting enzymes from *Bromelia hieronymi*, *W. coagulans*, *Solanum esculentum* and *Solanum macrocarpon* are stable proteins, remaining active after heating to 45, 70 and 70 °C, respectively (Bruno et al., 2010; Guiama et al., 2010; Naz et al., 2009). A milk-clotting enzyme called religiosin B, purified from *Ficus religiosa* stem latex, showed highest milk-clotting activity at temperatures of 55 and 60 °C (Kumari, Sharma, & Jagannadham, 2012).

Milk-clotting activity from *M. oleifera* flowers was highest after previous incubation of PP at pH 3.0 (Table 2) and lost of activity was detected when PP was previously incubated at pH values higher than 8.0. Calf rennet showed similar behaviour, acting better in acid than in alkaline reaction medium (Richardson, Nelson, Lubnow, & Schwarberg, 1967). Differently, the milk-clotting enzyme religiosin B showed highest clotting ability at pH 6.0 (Kumari et al., 2012).

High thermal stability and ability to work in a wide pH range are important criteria for the choice of proteases to be used in industrial processes (Vieille & Zeikus, 1996). In this sense, the

Table 1
Effect of heating of PP (precipitated protein fraction from *M. oleifera* flowers) on caseinolytic and milk-clotting activities.

Temperature (°C)	Activity (U)	
	Caseinolytic	Milk-clotting
Control	37.5 ± 2.5^{a}	1.9 ± 0.2^{A}
30	38.2 ± 2.3^{a}	1.9 ± 0.3^{A}
40	39.4 ± 1.1^{a}	1.8 ± 0.2^{A}
50	94.0 ± 1.0^{b}	2.1 ± 0.0^{B}
60	0	0.3 ± 0.1^{C}
70	0	0
80	0	0
90	0	0
100	0	0

One unit of caseinolytic activity on azocasein was defined as the amount of enzyme that gave an increase of 0.01 in absorbance. Different lowercase letters indicate significant differences at $p < 0.05$. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of skim milk within 180 min. Different uppercase letters indicate significant differences at $p < 0.05$. Control treatment corresponds to incubation of PP for 24 h at 28 °C.

Table 2
Effect of incubation of PP (precipitated protein fraction from *M. oleifera* flowers) at different pH values on caseinolytic and milk-clotting activities.

pH	Activity (U)	
	Caseinolytic	Milk-clotting
3.0	22.4 ± 1.8^{a}	2.1 ± 0.4^{A}
4.0	30.8 ± 1.0^{b}	1.9 ± 0.2^{B}
5.0	10.1 ± 1.2^{c}	1.75 ± 0.2^{B}
6.0	3.4 ± 1.0^{d}	1.8 ± 0.1^{B}
7.0	35.5 ± 2.8^{e}	1.8 ± 0.1^{B}
8.0	19.6 ± 1.0^{a}	1.7 ± 0.3^{B}
9.0	3.5 ± 0.0^{d}	0
10.0	7.1 ± 1.2^{f}	0
11.0	3.0 ± 1.0^{d}	0

One unit of caseinolytic activity on azocasein was defined as the amount of enzyme that gave an increase of 0.01 in absorbance. Different lowercase letters indicate significant differences at $p < 0.05$. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of skim milk within 180 min. Different uppercase letters indicate significant differences at $p < 0.05$.

Table 3
Effect of protease inhibitors on enzyme activities from PP (precipitated protein fraction from *M. oleifera* flowers).

Inhibitor	Activity (U)	
	Caseinolytic	Milk-clotting
Control	39.0 ± 1.2^{a}	2.0 ± 0.2^{A}
Pepstatin A	30.0 ± 1.1^{b}	0.8 ± 0.0^{B}
EDTA	37.7 ± 3.6^{a}	1.9 ± 0.2^{A}
E-64	36.0 ± 1.8^{a}	1.3 ± 0.1^{C}
PMSF	35.2 ± 4.1^{a}	1.5 ± 0.1^{C}

One unit of caseinolytic activity on azocasein was defined as the amount of enzyme that gave an increase of 0.01 in absorbance. Different lowercase letters indicate significant differences at $p < 0.05$. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of skim milk within 180 min. Different uppercase letters indicate significant differences at $p < 0.05$. Control treatment corresponds to PP activity in abscence of inhibitors.

milk-clotting enzymes present in PP are promising candidates for application in milk-clotting at an industrial large scale. Additionally, the traditional use of *M. oleifera* flowers in human diet, being eaten raw or after lightly blanched (Makkar & Becker, 1996), is an indicative of PP safety for use in cheese production.

The evaluation of enzyme activities from *M. oleifera* flowers in presence of protease inhibitors (Table 3) showed that the caseinolytic activity on azocasein was not significantly ($p > 0.05$) altered in presence of PMSF, while milk-clotting activity was significantly ($p < 0.05$) reduced, by as much as 25%. E-64 significantly ($p < 0.05$) inhibited only milk-clotting activity (by 30%), while pepstatin A significantly reduced ($p < 0.05$) caseinolytic and milk-clotting activities, by 25% and 57.5%, respectively. The results reveal that milk coagulation promoted by PP can be due to serine, cysteine and aspartic proteases. Milk-clotting agents belonging to these three classes of enzymes have been reported. Corrons, Bertucci, Liggieri, López, and Bruno (2012) reported the presence of serine proteases with caseinolytic and milk-clotting activities in latex of *Maclura pomifera* fruits. Also, it has been shown that religiosin B is a serine protease (Kumari et al., 2012). Cysteine proteases from *B. hieronymi* fruits with milk-clotting ability were also described (Bruno et al., 2010). Chymosin and milk-clotting enzymes from *C. cardunculus* flowers and *Strebus aspler* twigs are aspartic proteases (Heimgartner et al., 1990; Llorente, Brutti, & Caffini, 2004; Senthilkumar, Ramasamy, & Subramanian, 2006).

4. Conclusions

M. oleifera flowers contain caseinolytic and milk-clotting activities. The data showed that PP contains a mixture of aspartic, cysteine, serine and Ca^{2+}-dependent proteases. Caseinolytic and milk clotting activities showed slightly different sensitivities to pH treatment. A heat dependent activation of proteolytic activities from PP was also demonstrated. From the perspective of food treatment and engineering, PP is a new source of proteases with potential use for cheese production, since it promotes extensive hydrolysis of κ-casein and low degradation of α_s- and β-caseins.

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